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Syntrophomonas wolfei gen. nov. sp. nov., an Anaerobic, Syntrophic, Fatty Acid-Oxidizing Bacterium

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An anaerobic, nonphototrophic bacterium that β-oxidizes saturated fatty acids (butyrate through octanoate) to acetate or acetate and propionate using protons as the electron acceptor (H_2 as electron sink product) was isolated in coculture with either a non-fatty acid-degrading, H_2-utilizing *Desulfovibrio* sp. or methanogens. Three strains of the bacterium were characterized and are described as a new genus and species, *Syntrophomonas wolfei*. *S. wolfei* is a gram-negative, slightly helical rod with round ends that possesses between two to eight flagella laterally inserted along the concave side of the cell. It has a multilayered cell wall of the gram-negative type. The presence of muramic acid, inhibition of growth by penicillin, and increased sensitivity of the cells to lysis after treatment with lysozyme indicate that peptidoglycan is present in the cell wall. Cells of *S. wolfei* contain poly-β-hydroxybutyrate. Isoheptanoate was degraded to acetate, isovalerate, and H_2. Carbohydrates, proteinaceous materials, alcohols, or other tested organic compounds do not support growth. Common electron acceptors are not utilized with butyrate as the electron donor. Growth and degradation of fatty acids occur only in syntrophic association with H_2-using bacteria. The most rapid generation time obtained by cocultures of *S. wolfei* with *Desulfovibrio* and *Methanospirillum hungatei* is 54 and 84 h, respectively. The addition of Casamino Acids but neither Trypticase nor yeast extract stimulated growth and resulted in a slight decrease in the generation time of *S. wolfei* cocultured with *M. hungatei*. The addition of H_2 to the medium stopped growth and butyrate degradation by *S. wolfei*.

Three major metabolic groups of bacteria are essential for the complete anaerobic degradation of organic matter to CO_2 and CH_4 in ecosystems without light and low in exogenous electron acceptors other than CO_2 (6, 8, 22, 42). A complex of fermentative bacteria ferment the primary substrates to products such as saturated fatty acids, H_2, and CO_2. The terminal metabolic group includes the diverse species of methanogens per se (1) which perform the essential functions involving the catabolism of the large amounts of acetate produced by the other groups to CO_2 and CH_4 and rapidly utilize the H_2 produced to reduce CO_2 to methane.

The intermediate metabolic group, the obligate proton-reducing (H_2-forming) acetogenic bacteria (6, 29), is a complex of species involved in the following: (i) oxidation of alcohols such as ethanol to acetate and H_2 (8) or other corresponding carboxylic acid; (ii) β-oxidation of even-carbon-numbered fatty acids to acetate and odd-carbon-numbered fatty acids to acetate, propionate, and H_2 (Table 1); (iii) the decarboxylation of propionate to acetate, CO_2, and H_2 (4); and (iv) possibly other reactions (18). It was previously thought that certain methanogens degrade the fatty acids with the production of CH_4 rather than H_2 (28, 37). However, the discovery that *Methanobacillus omelianskii* was composed of a bacterium that grew and produced acetate and H_2 from ethanol only in syntrophic association with an H_2-using bacteria such as a methanogen (8) led to the idea that propionate and longer-chained fatty acids were catabolized by similar syntrophic associations. The isolation in coculture with a single H_2-using species of a bacterium that catabolizes the normal monocarboxylic, saturated, four- to eight-carbon fatty acids with acetate and H_2 or acetate, propionate, and H_2 as the products (Table 1) was the first direct evidence for the existence of a nonmethanogenic bacterium that anaerobically degrades any of the fatty acids without light or sulfate, nitrate, or similar electron acceptors (29). The isolation of the fatty acid-oxidizing

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TABLE 1. *Proposed reactions involved in the catabolism of fatty acids by S. wolfei*[a]

Fatty acids	Reaction
Even-numbered	
$CH_3CH_2CH_2COO^-$	$+ 2H_2O \leftrightharpoons 2CH_3COO^- + 2H_2 + H^+$
$CH_3CH_2CH_2CH_2CH_2COO^-$	$+ 4H_2O \leftrightharpoons 3CH_3COO^- + 4H_2 + 2H^+$
$CH_3CH_2CH_2CH_2CH_2CH_2CH_2COO^-$	$+ 6H_2O \leftrightharpoons 4CH_3COO^- + 6H_2 + 3H^+$
Odd-numbered	
$CH_3CH_2CH_2CH_2COO^-$	$+ 2H_2O \leftrightharpoons CH_3CH_2COO^- + CH_3COO^- + 2H_2 + H^+$
$CH_3CH_2CH_2\ CH_2CH_2CH_2COO^-$	$+ 4H_2O \leftrightharpoons CH_3CH_2COO^- + 2CH_3COO^- + 4H_2 + 2H^+$
Branched-chained	
$CH_3\text{-}CHCH_2CH_2\ CH_2COO^-$	$+ 2H_2O \leftrightharpoons CH_3\text{-}CHCH_2COO^- + CH_3COO^- + 2H_2 + H^+$
CH_3	CH_3

[a] Calculated from the fermentation products produced by *S. wolfei* in coculture with *M. hungatei* (Table 2; reference 29) and from the following equation: $4H_2 + HCO_3^- + H^+ \rightleftharpoons CH_4 + 3H_2O$ (38). The arrows represent the points where cleavage of two-carbon units would occur during β-oxidation.

bacterium was difficult because of thermodynamics of the reaction and the necessity of an extremely low partial pressure of H_2 via coculture with the H_2-utilizing bacterium, as previously discussed (29, 42).

In this report we further characterize this bacterium (29) and propose that it be named *Syntrophomonas wolfei* gen. nov. sp. nov. A bacterium, *Syntrophobacter wolinii*, which degrades propionate to acetate and, presumably, CO_2 and H_2 in coculture with an H_2-using *Desulfovibrio* sp. was recently described (4). A brief report of *S. wolfei* appeared previously (M. J. McInerney and M. P. Bryant, Abstr. Annu. Meet. Am. Soc. Microbiol. I62, p. 94, 1980).

MATERIALS AND METHODS

Sources of organisms. *Methanospirillum hungatei* JF-1 (17), *Methanobacterium bryantii* (*Methanobacterium* strain MoH; 1), *Desulfovibrio* strain G11 (29), an H_2-utilizing sulfate reducer, and the Göttingen and Urbana strains of *S. wolfei* in coculture with *M. hungatei* (29) were obtained from our culture collection (Department of Dairy Science, University of Illinois).

The Boneyard strain of *S. wolfei* was isolated in coculture with *Desulfovibrio* strain G11 from the sediment of a Winogradsky column initiated with sediments obtained from the Boneyard Creek, Urbana, Ill., by serial dilution and inoculation of roll-tube medium as previously described (29). A coculture of the Göttingen strain of *S. wolfei* with *M. bryantii* was obtained by serially diluting a coculture of the Göttingen strain with the *Desulfovibrio* strain in anaerobic dilution solution and inoculating butyrate roll-tube medium as described (29) but containing a large inoculum of *M. bryantii* rather than *M. hungatei*.

Media and conditions of cultivation. The anaerobic techniques for the preparation and use of media were those of Hungate as modified (5).

The basal medium contained 5% (vol/vol) of rumen fluid, minerals, B vitamins, cysteine-sulfide reducing agents, $NaHCO_3$, and 80% N_2–20% CO_2 gas phase, final pH 7.2 (29). The minimal medium was the same composition as the basal medium except that the B vitamin solution and clarified rumen fluid were deleted. Solid medium for roll tubes and slants was prepared by including 2.0% and 1% (wt/vol) of agar (Difco), respectively, in the basal medium. For 300-ml cultures, 500-ml nephelo culture flasks fitted with black rubber stoppers were used. The 700-ml and 1,500-ml cultures were prepared in 1- and 2-liter reagent bottles fitted with rubber stoppers. After the medium cooled, the negative pressure inside the bottle was equalized by inserting through the stopper a sterile 20-gauge needle fitted to the syringe of the gassing apparatus (5) through which flowed an 80% N_2–20% CO_2 gas mixture. The appropriate amount of $NaHCO_3$ and cysteine-Na_2S solutions were each added to the cooled sterile medium except for the 700-ml and 1,500-ml cultures, where $NaHCO_3$ was added as a solid (0.35 g/100 ml) before autoclaving. These latter cultures were allowed to equilibrate for 1 to 2 days before inoculation for the medium to reduce and equilibrate with the gas phase so that the pH was 7.2. For enrichment cultures, 2.5% (wt/vol) $Na_2S \cdot 9H_2O$ solution (7) replaced the cysteine-Na_2S solution. Each medium was supplemented, as indicated, with sodium butyrate and Na_2SO_4 for cultures containing *Desulfovibrio* strain G11, from sterile anaerobic stock solutions, or by including thesecompounds in the medium before autoclaving.

The various strains of bacteria were maintained in slant culture and grown in liquid culture (29). A 15-ml culture was used to inoculate the 300-ml or larger volume cultures.

To determine if *S. wolfei* would grow alone if the H_2 in the gas phase was continually removed, a 500-ml reagent bottle containing 250 ml of the butyrate basal medium with only 2 mM Na_2SO_4 was inoculated with 10 ml of a coculture of the Göttingen strain of *S. wolfei* with *Desulfovibrio* strain G11. The gas phase of the culture (on a shaker) was recycled, using a diaphragm pump, through a column containing hot copper oxide wire to remove H_2, and hot copper wire (5) to remove

any residual O_2 before reentering the culture.

All incubations were at 35°C with cultures held in a vertical position except for liquid cultures which had H_2 in the atmosphere. These were incubated in a slanted position on a reciprocal shaker (8).

Culture purity. Cultures were routinely checked for purity by examining wet mounts and Gram stains and by inoculating 0.1% glucose-Trypticase soy broth (BBL Microbiology Systems) which was incubated under both aerobic and anaerobic conditions. The medium allowed no growth of either organism in the methanogenic coculture but should allow growth of most contaminating bacteria.

Substrate utilization. Tests for the utilization of energy sources were made by inoculating the basal medium, with and without the compound added at a final concentration of 0.2%, with the coculture of *S. wolfei* with *M. hungatei* (29). Tests for the utilization of a compound as an electron acceptor were performed as described above except that 18 mM butyrate was added to serve as the electron donor.

Fermentation balances. The fermentation products produced from butyrate by the Boneyard strain of *S. wolfei* in coculture with *Desulfovibrio* strain G11 or from isoheptanoate by the Göttingen strain with *M. hungatei* were determined by inoculating each of three tubes with and without the substrate and incubating the tubes until growth ceased. Liquid samples were withdrawn from each tube at the start and end of the incubation period, centrifuged (12,000 × *g*, 10 min, 4°C) and frozen at −20°C until analyzed. The amount of acids and CH_4 produced by the cultures was corrected for the amount found in cultures without added substrate.

Effect of H_2 on growth of *S. wolfei*. Each of four tubes containing the basal medium with butyrate was inoculated with a coculture of the Göttingen strain with *M. hungatei*. Two of these tubes were gassed with an 80% N_2–20% CO_2 gas mixture, and the other two were gassed with 80% H_2–20% CO_2. The tubes were incubated with shaking for 4 days at which time the gas phase was replaced daily for the next 4 days with the same gas phase that the tube had received at the start of the experiment. A 4-ml volume from each tube was then withdrawn and centrifuged (12,000 × *g*, 10 min, 4°C). The supernatant fluid was decanted and frozen at −20°C until the butyrate concentration was determined. The pellet was resuspended in 1 ml of a 10% formaldehyde (vol/vol)–0.85% NaCl (wt/vol) solution, and the numbers of each bacterium were estimated using a Petroff-Hausser counting chamber (40).

Effect of penicillin on growth rate and yield. Tubes containing the butyrate basal medium had an 80% N_2–20% CO_2 gas phase for those inoculated with the coculture of the Göttingen strain with *M. hungatei* or an 80% H_2–20% CO_2 gas phase for those inoculated with *M. hungatei* alone. Stock solutions of penicillin G (Sigma Chemical Co., St. Louis, Mo.) were prepared in anaerobic dilution solution (29) so that the addition of 0.1 ml to tubes gave final concentrations of 10, 20, or 100 μg/ml. Control tubes received 0.1 ml of anaerobic dilution solution. The 80% H_2–20% CO_2 gas phase was replaced daily.

Determination of muramic and diaminopimelic acids. Cell pellets obtained from each of two 300-ml cocultures of the Göttingen strain with *M. hungatei* were anaerobically washed twice by centrifugation (4,160 × *g*, 20 min, 4°C) using a 50 mM K_2HPO_4 buffer (pH 7.2) containing in percent (wt/vol): NaCl, 0.9; $MgCl_2 \cdot 6H_2O$, 0.25; resazurin, 0.0001; $Na_2S \cdot 9H_2O$, 0.025; dithiothreitol, 0.06. The manipulations were performed in an anaerobic chamber (2). Each washed cell suspension was lyophilized to dryness, acid hydrolyzed, and analyzed for amino acids and amino sugars (21).

Isolation of poly-β-hydroxybutyrate. The combined cell pellet obtained from six 700-ml cocultures of the Göttingen strain with *M. hungatei* was washed three times by centrifugation (12,000 × *g*, 10 min, 4°C) using distilled water. The poly-β-hydroxybutyrate (PHB) material was isolated from the cell suspension using the procedure of Herron et al. (20). The final residue was dissolved in about 1 ml of warm chloroform and transferred to a tared aluminum weighing pan. The chloroform was evaporated by heating at 45°C, and the pan was reweighed. As a control, about 0.2 g (wet weight) of cells of *M. hungatei* grown alone in the butyrate basal medium with an 80% H_2–20% CO_2 gas phase was extracted.

Electron microscopy. A 50-ml sample of a coculture of the Göttingen strain with *M. hungatei* was prefixed by the addition of 100 ml of a 5% (vol/vol) glutaraldehyde–0.067 M cacodylate buffer (pH 7.2) with 0.15% ruthenium red. Another 50-ml sample was fixed as above except that the buffer did not contain ruthenium red. Each suspension was incubated for 2 h at room temperature and then centrifuged (12,000 × *g*, 10 min, 23°C). The pellet was washed twice by resuspension of the pellet in the respective buffer for 20 min followed by centrifugation as above. Each cell suspension was fixed with osmium tetroxide, dehydrated, embedded, and sectioned (11). Sections were examined using an AEI 801 electron microscope.

Isolation and determination of base ratio of DNA. The deoxyribonucleic acid (DNA) was isolated using the procedure of Marmur (29) from about 3 and 0.8 g (wet weight) of cells of the Göttingen strain cocultured with *M. hungatei* and *Desulfovibrio* strain G11, respectively, obtained from several 700-ml and 1,500-ml cultures. A portion of the DNA was centrifuged to equilibrium in cesium chloride using a Beckman model E ultracentrifuge with *Micrococcus luteus* DNA (ρ, 1.7039 g·cm^{-3}; a gift from W. E. Balch) as the reference standard (35). The guanine plus cytosine (G+C) content of DNA was calculated by the method of Schildkraut et al. (35).

Other methods. Growth was determined spectrophotometrically (29). The gas volume of a culture was measured using a manometer (8), and CH_4 and H_2 were determined by gas chromatography (42). The amount of protein of a cell suspension was determined by the colorimetric method (26) with bovine serum albumin (Pentex Biochemicals, Kankakee, Ill.) as a standard after digesting the sample in 0.1 N NaOH at 70°C for 10 min. The concentration of fermentation acids was determined by the butyl ester-gas chromatographic method (34). Isoheptanoic acid was identified and quantitated using the butyl ester prepared

from a known concentration of isoheptanoic acid (Sigma Chemical Co., St. Louis, Mo.) as the standard.

RESULTS

Presence in ecosystems. The Boneyard strain was morphologically identical to the two previously described strains of *S. wolfei* from anaerobic digestor sludge (29) and produced 198.1 ± 4.2 μmol of acetate per 100 μmol of butyrate degraded (99% carbon recovery) when cocultured with *Desulfovibrio*. Also, microscopic analysis of butyrate-degrading enrichments initiated from sediments of two different lagoons in Urbana, Ill., and from bovine rumen fluid (30) suggested that the major butyrate-degrading bacteria in these enrichments were morphologically identical to the strains of *S. wolfei* already isolated.

Energy sources and fermentation products. In addition to other fatty acids (29), isoheptanoate supported growth and methane production of the Göttingen strain with *M. hungatei*. Isoheptanoate was degraded to acetate, isovalerate, and methane by this coculture (Table 2). Additional compounds tested and shown not to support growth of the Göttingen strain with *M. hungatei* included δ-amino-*n*-butyrate, butyraldehyde, isocaproate, glucose, propionate, pyruvate, and Trypticase. Manganese oxide, methyl viologen, palladium chloride, phenosafranin, tetrazolium chloride, and trimethylamine-*N*-oxide were not used as electron acceptors with butyrate as the electron donor.

H_2 production and inhibition. *S. wolfei* was cocultured with *M. bryantii* strain MoH, another methanogen that uses only H_2-CO_2. The fact that CH_4 was produced from butyrate by this coculture shows that *S. wolfei* produced H_2 from butyrate (Table 3). Formate was not metabolized to CH_4 which excludes the possibility that the coculture of *S. wolfei* with *M. bryantii* contained a contaminant which could metabolize formate to H_2 and CO_2 and which could then be used by *M. bryantii*. Other data (not shown), using slant cultures of *S. wolfei* and *M. bryantii* which grow much better than liquid cultures, showed that the amount of CH_4 produced from butyrate by the coculture was almost equal to that expected if all the reducing equivalents formed in the degradation of butyrate by *S. wolfei* were used to produce H_2. In addition, the Göttingen strain of *S. wolfei* grew when cocultured with *Methanobrevibacter arboriphilus* (*Methanobacterium arbophilicum*) strain AZ, another methanogen which uses only H_2-CO_2 for methanogenesis (29).

An initial partial pressure of 0.8 atm (8×10^4 Pa) of H_2 completely inhibited the growth and butyrate degradation by the Göttingen strain in coculture with *M. hungatei* (data not shown).

An experiment was performed to determine if *S. wolfei* would grow alone with butyrate as the energy source when the gas phase was continually recycled after passage through hot copper oxide filings to remove H_2; however, no growth occurred.

Growth rates and some nutritional features of *S. wolfei*. The Göttingen strain in coculture with *Desulfovibrio* strain G11 grew

TABLE 2. *Fermentation products formed from isoheptanoate by the Göttingen strain of S. wolfei in coculture with M. hungatei*[a]

Product	μmol/100 μmol of isoheptanoate fermented
Acetate	77.4 ± 1.5[b]
Isovalerate	81.1 ± 5.6
CH_4	47.4 ± 4.0
CO_2[c]	−47.4

[a] The initial isoheptanoate concentration was 10.2 mM. See text for experimental details. Other common bacterial fermentation products such as formate, valerate, caproate, lactate, succinate, and H_2 were not detected. Percent C recovered, 81.3; percent H recovered, 81.8. (H is "available hydrogen" calculated by the method of Barker [3].)

[b] Mean ± standard deviation of the values obtained from three individual cultures after 90 days of incubation.

[c] Calculated as equal to the amount of CH_4 formed.

TABLE 3. *Growth and CH_4 production from butyrate by the Göttingen strain of S. wolfei in cocultures with M. bryantii or M. hungatei*[a]

Substrate	Inoculum[b]	Growth (OD)[c]	CH_4 (μmol)
Butyrate	*S. wolfei* + JF1	0.12 (6)	40
	S. wolfei + MoH	0.01 (8)	13
	MoH	0.01 (2)	0
Formate	*S. wolfei* + JF1	0.05 (2)	59
	S. wolfei + MoH	0.01 (8)	0
	MoH	0.01 (2)	0

[a] Each tube containing the basal medium was inoculated with 1 ml of culture and incubated for 70 days. Concentrations of the sodium salts of butyrate and formate were 18 and 28 mM, respectively, as indicated. Values are means of values obtained from two individual cultures and are corrected for the small amount of growth and CH_4 production that occurred in cultures without added substrate.

[b] The inoculum cultures were *S. wolfei* with *M. hungatei* (JF1), *S. wolfei* with *M. bryantii* (MoH), and MoH alone.

[c] OD, Maximum optical density obtained, with the number in parentheses representing the days required to reach that value.

with a generation time of 54 h compared to a 90-h generation time in coculture with *M. hungatei* (Fig. 1). Increasing the butyrate concentration from 10 to 20 mM resulted in greater growth but did not affect the growth rate. Higher concentrations of butyrate (50 mM) decreased the amount of growth. Generation times of 90 and 180 h were obtained by the coculture of the Göttingen strain with *M. hungatei* with valerate and caproate, respectively, as the energy source. Similar amounts of growth were obtained with each of the three energy sources. The coculture of the Göttingen strain with *M. hungatei* grew better in the butyrate basal medium which contained rumen fluid and B vitamins than in the butyrate minimal medium, and the further addition of Casamino Acids, but not other materials, further stimulated growth (Table 4).

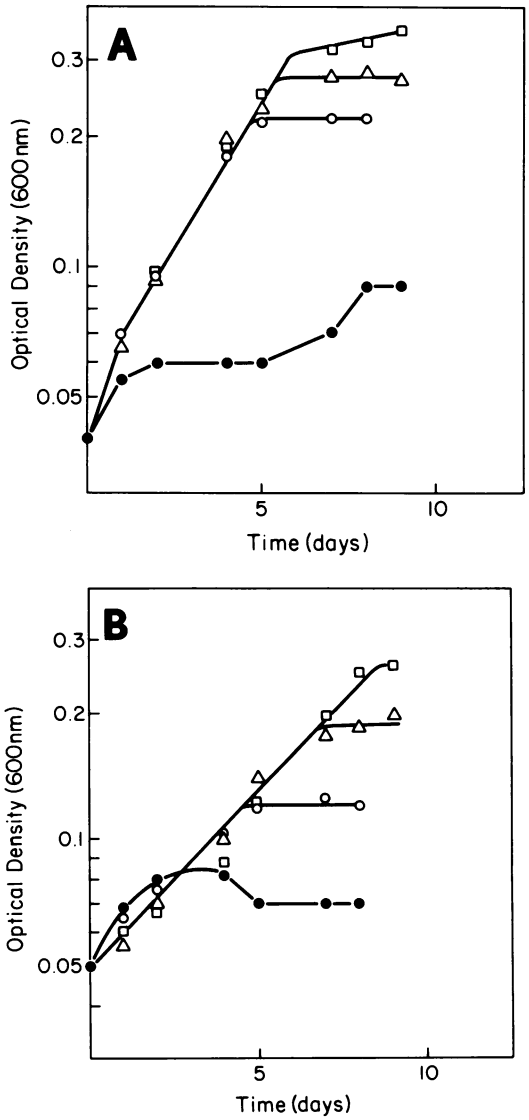

FIG. 1. *Effect of butyrate concentration on the growth of the Göttingen strain of S. wolfei in coculture with Desulfovibrio (A) or M. hungatei (B). Each of three tubes containing the basal medium with the indicated butyrate concentration and 21 mM Na_2SO_4 when the Desulfovibrio was present was inoculated with 0.2 ml of the coculture. Values are means of the values obtained from three individual cultures. Symbols: ●, none; ○, 10 mM; □, 20 mM; △, 50 mM sodium butyrate added.*

Ultrastructure. The cell wall of the Göttingen strain was about 50 nm wide and was a complex example of the gram-negative cell-wall type (Fig. 2). It has a lightly stained but resolvable outer membrane with an irregular contour and a well-defined double-tracked cytoplasmic membrane. The periplasmic space contained a lightly stained double-tracked layer and a homogeneous electron-dense layer adjacent to the cytoplasmic membrane. These structures are more discernible in partially lysed cells. The cytoplasm contained electron-translucent and electron-dense granules and a rectangular core-like structure. Ruthenium red-stained material was present in the intercellular space but did not seem to be closely associated with the cells.

TABLE 4. *Growth rates and yield obtained by the Göttingen strain of S. wolfei in coculture with M. hungatei in minimal medium or basal medium with or without nutrient supplementation*

Expt[a]	Medium	Generation time (h)[b]	Yield[c]
1	Minimal	156	0.19
	Basal	89	0.28
2	Basal alone	84	0.15
	Basal + Casamino Acids (0.2% [wt/vol])	78	0.14
	Basal + Trypticase (0.2% [wt/vol])	84	0.16
	Basal + yeast extract (0.2% [wt/vol])	87	0.15

[a] Two tubes of each medium with 18 mM sodium butyrate were inoculated with 0.2 ml of the coculture and incubated for 11 days. In experiment 1, the inoculum had been grown in the butyrate minimal medium for five transfers. In experiment 2, the inoculum was grown in the butyrate basal medium.

[b] Each generation time was estimated from a growth curve plotted using the means of the values obtained from two individual cultures.

[c] Maximal optical density obtained. Each value is a mean of the values obtained from two individual cultures and is corrected for the initial optical density of the culture.

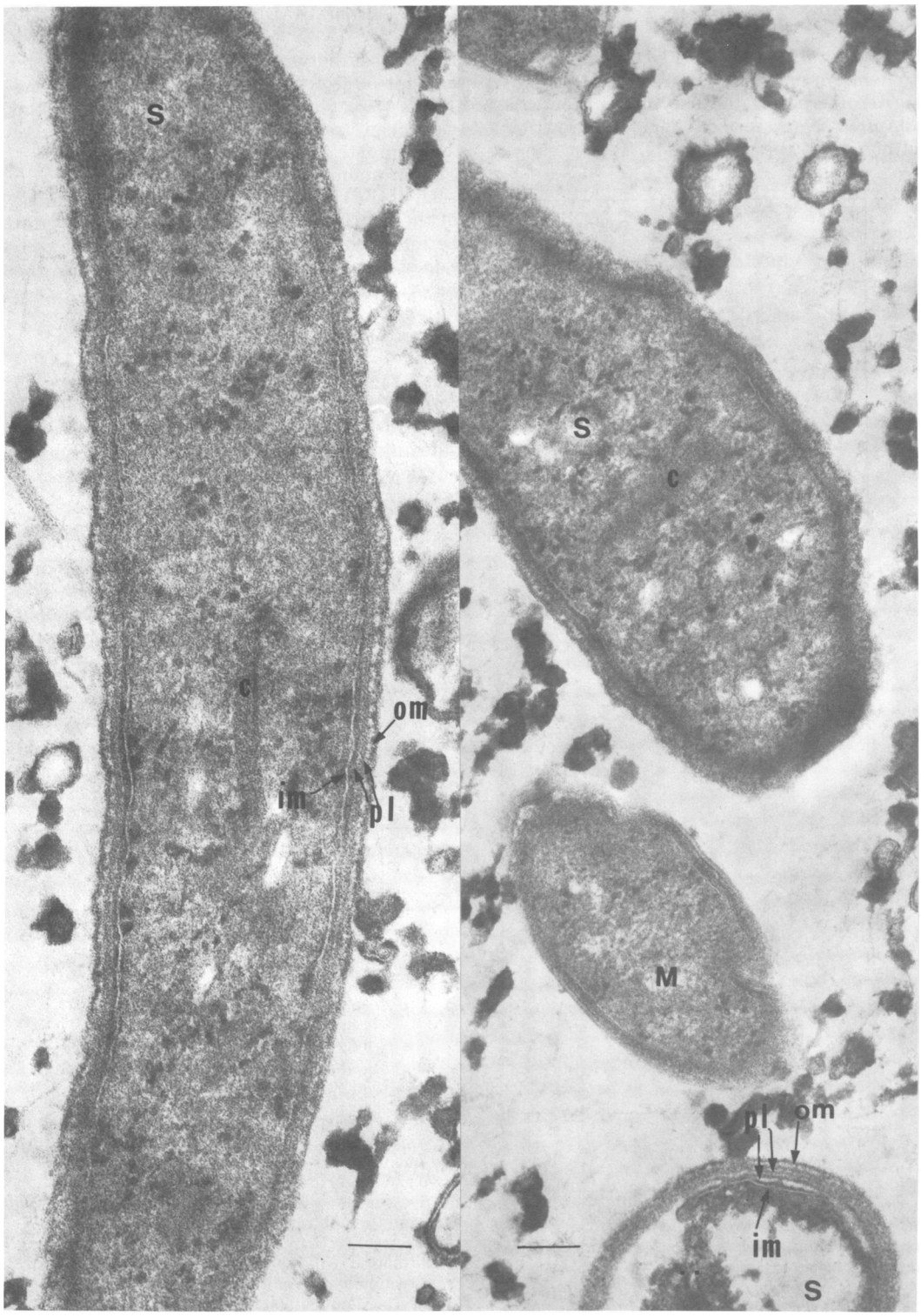

FIG. 2. *Electron micrograph of a section of ruthenium red-stained material from the coculture of the Göttingen strain of S. wolfei (S) with M. hungatei (M). Note the resolvable outer membrane layer (om), the double-tracked cytoplasmic membrane (im), the periplasmic layers (pl), as well as the core-like structure (c). Bars represent 0.1 μm.*

Evidence for peptidoglycan. Penicillin G concentrations of 10 and 20 μg/ml resulted in a 50% decrease in the growth rate of the coculture of the Göttingen strain with *M. hungatei* compared to cultures that did not receive penicillin. Concentrations of 100 μg/ml completely inhibited the growth of the coculture, but the growth of *M. hungatei* alone was not affected. Cells of *S. wolfei* were swollen and rounded in cultures with penicillin, whereas cells of *M. hungatei* were unaffected.

The addition of 1 mg of lysozyme (Sigma) per ml stimulated the lysis of cells of *S. wolfei* but not of *M. hungatei* (data not shown).

The analysis of acid hydrolysates obtained from two separate cocultures of the Göttingen strain with *M. hungatei* showed peaks corresponding to muramic and *meso*-diaminopimelic acids. The presence of muramic acid was confirmed by heating another sample in HCl at 120°C for 16 h which leads to the complete destruction of amino sugars (21). The analysis of this sample showed that the muramic acid peak had disappeared but the amino acid peaks remained unchanged. Since *M. hungatei* does not contain muramic or *meso*-diaminopimelic acids (21), these compounds must have been present in the cell wall of *S. wolfei*.

PHB in cells of *S. wolfei*. Smears of the cocultures of the three strains of *S. wolfei* showed that the cells contained sudanophilic granules. These granules did not stain when procedures to detect spores, glycogen, or volutin were used (31). Neither the cells of *M. hungatei* nor *Desulfovibrio* strain G11 present in these cocultures nor the cells of the H_2 utilizer grown alone in the butyrate basal medium with an 80% H_2–20% CO_2 gas phase contained these granules. About 46 μg of PHB-like material per mg of protein was obtained from cells of the Göttingen strain cocultured with *M. hungatei*. No PHB-like material was recovered from cells of *M. hungatei* (57 mg of protein). The PHB-like material was resistant to digestion by 5% sodium hypochlorite, was insoluble in water, 95% alcohol, and ether, and was soluble in chloroform. This material had an ultraviolet absorption spectrum similar to pure β-hydroxybutyrate (Sigma Chemical Co.), and the ultraviolet absorbance of both substances increased greatly with a maximum at 234 nm upon heating in concentrated sulfuric acid (Fig. 3). Heating in concentrated sulfuric acid quantitatively converts PHB to crotonic acid (24), and the ultraviolet absorbance maximum of crotonic acid in concentrated sulfuric acid was 235 nm (36). Rehydration of a heated sample of PHB by the addition of an equal volume of water shifted the ultraviolet absorbance maximum to 210 to 215 nm (20). The solubility properties, resistance to digestion by 5% sodium hypochlorite, and the ultraviolet absorbance properties indicated that this isolated material contained PHB.

DNA. Equilibrium density gradient ultracentrifugation of DNA extracted from the coculture of *S. wolfei* with *M. hungatei* showed only one band corresponding to a G+C content of 44.8 mol%. The G+C content of *M. hungatei* is 45 mol% (17). Similar analysis of the DNA extracted from the coculture of the Göttingen strain with *Desulfovibrio* strain D11 showed only one band corresponding to a G+C content of 57.5 mol% which was assumed to be the DNA of the *Desulfovibrio* (32). Increasing the DNA concentration 10-fold resulted in a dispersion of the DNA throughout the gradient, and no distinct bands were observed.

DISCUSSION

The isolation via coculture with H_2-using bacteria of *S. wolfei* (29) and of *S. wolinii* (4) provided direct evidence both for the existence of the fatty acid-catabolizing metabolic group of bacteria called the obligate proton-reducing (H_2-forming) acetogenic bacteria (6) and for its essential involvement in the complete anaerobic degradation of organic matter to CO_2 and CH_4 (8, 22, 43).

The maximum generation time of *S. wolfei* on butyrate and in coculture with *M. hungatei* at 35°C fits well with the generation time (about 84 h) found by McCarty and his students (28) to be necessary for degradation of butyrate in semicontinuous mixed cultures degrading municipal raw sewage digestor sludge. The generation time was considerably shorter (54 h) when *S. wolfei* was cocultured with *Desulfovibrio* where sulfide rather than methane served as the final electron sink product. The possible relationship of these differing generation times to differing changes in free energy of the reactions involved (i.e., butyrate catabolism to acetate and H_2, and H_2 oxidation coupled to CO_2 or sulfate reduction) and to affinities of the H_2-oxidizing species for H_2 was previously discussed (4).

It was also apparent that neither *M. bryantii* (this study) nor *M. arboriphilus* (29) allowed as good growth on butyrate in coculture with *S. wolfei* as did *M. hungatei*, the dominant H_2-using organism found in our butyrate or propionate enrichments from nonmarine natural ecosystems (4, 40; this study), other than the rumen where an acetate and H_2-using *Methanosarcina* dominated (30).

S. wolfei is the only bacterium known to obtain energy for growth from the anaerobic degradation of the normal monocarboxylic, saturated, four- to eight-carbon fatty acids and from

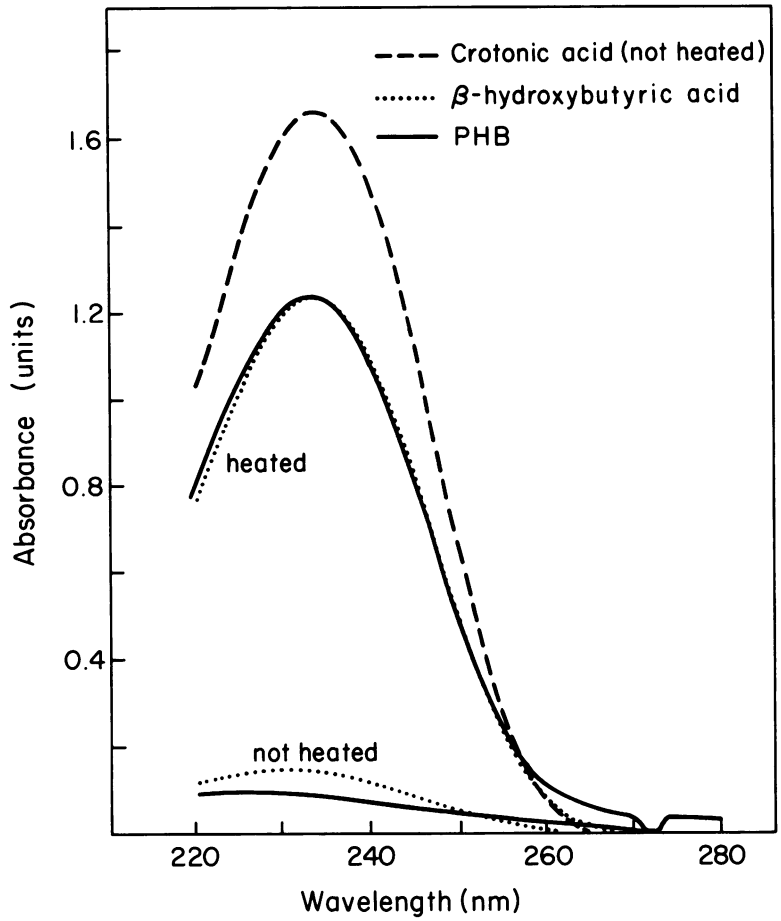

FIG. 3. *Ultraviolet absorbance spectra of crotonic acid, β-hydroxybutyric acid, and the isolated PHB material isolated from the coculture of the Göttingen strain of S. wolfei with M. hungatei. Each mixture contained 9 ml of concentrated sulfuric acid and 40 μg of the isolated PHB material or of crotonic or β-hydroxybutyric acids with enough distilled water to bring the volume to 10 ml. The chloroform used to dissolve the isolated PHB material was allowed to evaporate before the addition of the other components. The spectra of mixtures containing either the isolated PHB material or β-hydroxybutyric acid were measured before and after heating at 100°C for 10 min. Spectra were obtained using a Varian Techtron model 635 split-beam spectrophotometer with concentrated sulfuric acid in the reference curve.*

isoheptanoate, with H_2 and acetate, acetate and propionate, or acetate and isovalerate being the products (Table 2; 29) in the absence of light energy or electron acceptors such as sulfate and nitrate. The products produced from the various fatty acids are consistent with a β-oxidation mechanism for the degradation of fatty acids. Hydrogen has been shown to be the electron sink product by coculturing *S. wolfei* with methanogens that use only H_2 for growth and methanogenesis (29; this paper).

Only a limited number of compounds support growth of *S. wolfei* and, as yet, we have been unable to document its use of fatty acids in the absence of another bacterium to utilize the H_2 produced. H_2, as 80% of the culture gas phase, stopped its growth and butyrate oxidation, and much less H_2 would strongly inhibit the beta oxidation and H_2 production (less than about 10 nM H_2 calculated from change in free energy of the reaction with appropriate concentrations of reactants and products [38, 43]).

It may be that methods will be found to grow *S. wolfei* alone by effective removal of H_2 from cultures. However, the strategy used in the present experiments, starting with *S. wolfei* plus *Desulfovibrio* inoculated into medium without sulfate and rapidly sparging with recirculated 80% N_2–20% CO_2 gas phase (maintained O_2-free by passage through a hot copper column to

remove any O_2 entering the system and maintained with low H_2 by passage through a hot copper oxide column to remove H_2 produced), did not allow growth. It is possible that something toxic to growth was generated via the treatments of the recirculated gas. However, the two organisms maintained their motility, and visual reduction of a small amount of copper oxide to copper suggested that a small amount of H_2 was produced.

The presence of PHB-like material in *S. wolfei* was not expected as PHB has not been detected in non-phototrophic anaerobes (13, 14) except for *Clostridium botulinum* type E (16) and mutants of *Rhodospirillum rubrum* grown heterotrophically in the dark (39). Two possible pathways of PHB synthesis might occur in *S. wolfei*. It could be synthesized from β-hydroxybutyryl·coenzyme A (assuming a pathway of β-oxidation exists as found in other bacteria) formed during the catabolism of butyrate or via the condensation of acetyl-coenzyme A units as in some other bacteria (14). If the former were the case, then poly-β-hydroxyvalerate would be expected to be formed when valerate serves as the energy source. PBH may function as a carbon and/or energy reserve as it does in other bacteria (13, 14). Another possibility is that, by the polymerization of β-hydroxybutyryl·coenzyme A, the equilibria of reactions involved in earlier steps in butyrate catabolism are shifted towards the production of β-hydroxybutyryl·coenzyme A. This might make the production of H_2 from electrons generated in the dehydrogenation of butyryl·coenzyme A to crotonyl·coenzyme A ($E_0^1 = -15$ mV) (15) energetically more favorable. The E_0^1 of the H^+/H_2 couple is −414 mV (38).

The arrangement of flagella in *S. wolfei* is unusual (29) as only *Selenomonas* (23) and *Pectinatus cerevisiiphilus* (25) have similar arrangements. However, the points of insertion of flagella in *Selenomonas* are much closer together and more centrally located. Both *Selenomonas* and *Pectinatus* have more flagella per cell (10 to 23 or more) than *S. wolfei*. The size and ultrastructure of the flagella closely resemble those of the genus *Selenomonas* (10, 23).

S. wolfei has an unusual multilayered cell wall, but the presence of resolvable inner and outer membranes indicates that it has a gram-negative cell-wall type (12, 19). The detection of muramic and *meso*-diaminopimelic acids and penicillin sensitivity of the organism confirms the presence of peptidoglycan in the cell wall and shows that *S. wolfei* is probably not a member of the *Archaebacteria* (41).

It was not possible to determine the G+C content of the DNA of *S. wolfei* as it was either very similar to *M. hungatei* or *Desulfovibrio* or was present in too small an amount in the cocultures to allow its detection with the methods used. Perhaps selective methods could be found to separate the DNA of *S. wolfei* from that of the H_2-using cocultured species.

S. wolfei differs from all of the other described bacterial species and genera except *S. wolinii* (4) in its ability to anaerobically oxidize fatty acids with protons serving as electron acceptor. It differs from *S. wolinii* in its morphology, in its substrate specificity, and in having flagella. It is similar to the S organism (8, 33) in being a gram-negative anaerobe that produces H_2 and acetate and in its syntrophic dependence on H_2-using bacteria. It differs from the S organism in not utilizing alcohols, pyruvate, or acetaldehyde, in using fatty acids, and in arrangement and size of flagella. A similar bacterium was probably present in the "highly enriched" culture called *Methanobacterium suboxydans* (37) which utilized energy sources similar to the present syntrophic bacterium. The isolation of cocultures of *S. wolfei* with several different methanogens and with *Desulfovibrio* strain G11 confirmed the purity of the cocultures.

The type species of the genus, *S. wolfei*, is named to honor R. S. Wolfe for his devotion towards the understanding of the biology of anaerobic bacteria. The assignment of these new taxa to higher taxa is not feasible at this time. A combined description of these taxa follows.

Syntrophomonas wolfei gen. nov. sp. nov., syn. tro.pho.mon.'as, Gr. adj., *syn* together with; Gr. n., *trophos* one who feeds; Gr. n., *monas* a unit, monad; M.L. fem.n. *Syntrophomonas*, monad which feeds together with (another species). Wolf'.e.i. M.L. gen. n. *wolfei* of Wolfe.

Nonsporing, gram-negative, slightly helical rods, 0.5 to 1.0 by 2.0 to 7.0 μm with slightly tapered rounded ends. Most cells occur singly or in pairs with helical chains of three or more often observed. Multiplication by binary fission. Cells possess two to eight flagella with a diameter of about 20 nm that are laterally inserted in a linear fashion on the concave side of the cell about 130 nm or more apart. Under most conditions, cells usually exhibit only a sluggish twitching motility. Cells have an unusual multilayered gram-negative wall. Muramic and *meso*-diaminopimelic acids are present, and the organism is sensitive to penicillin. PHB is present.

Surface colonies in roll tubes of *S. wolfei* cocultured with methanogens are smooth, convex, and circular, with entire edges and black in color when the *Desulfovibrio* species is the H_2-utiliz-

ing bacterium. Colonies form in 3 to 20 weeks and may reach 2 to 3 mm in diameter. Subsurface colonies are lenticular.

S. wolfei is chemoorganotrophic. It anaerobically β-oxidizes fatty acids with protons serving as the electron acceptor. Butyrate, caproate, and caprylate are degraded to acetate and H_2; valerate and heptanoate are degraded to acetate, propionate, and H_2; and isoheptanoate is degraded to acetate, isovalerate, and H_2. Carbohydrates, proteinaceous materials, alcohols, or other organic compounds do not support growth. Common electron acceptors such as fumarate, malate, nitrate, oxygen, sulfate, sulfite, sulfur, thiosulfate, or others are not utilized with butyrate as the electron donor. Growth and degradation of fatty acids occur only in syntrophic association with H_2-utilizing bacteria and are stimulated by factors in rumen fluid and/or B vitamin mixture.

Isolated from anaerobic environments, such as aquatic sediments, sewage digestor sludge, and rumen digesta, where organic matter is degraded with CO_2 and CH_4 as major products.

The Göttingen strain is the type strain of the genus and species.

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